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Effects of Nitrogen Limitation on the Growth and Composition of Unicellular Algae in Continuous Culture

B. RICHARDSON, D. M. ORCUTT, H. A. SCHWERTNER, CARA L. MARTINEZ, AND HAZEL E. WICKLINE

Environmental Systems Division, USAF School of Aerospace Medicine, Aerospace Medical Division (AFSC), Brooks Air Force Base, Texas 78235

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Since fat accumulation takes place in many algae as a response to exhaustion of the nitrogen supply, it has been suggested that this may provide a means of enhancing the potential food value of algae. To test this possibility, chemostatic continuous cultures of *Chlorella sorokiniana* and *Oocystis polymorpha* were subjected to successive reductions in influent nitrogen. As cellular nitrogen content decreased from about 10 to 4%, oxygen evolution, carbon dioxide uptake, chlorophyll content, and tissue production were drastically reduced, but total lipid content was essentially unchanged. Caloric values and C, H, and N analyses suggested a moderate increase in carbohydrate content, but gas chromatographic analyses revealed no significant qualitative or quantitative changes in the fatty acid fraction. In batch-cultured cells, nitrogen could be reduced to 3% of dry weight, causing a concomitant increase in total fatty acids and pronounced changes in the composition of the fatty acid fraction. These results suggest that cellular nitrogen must fall to approximately 3% of dry weight before appreciable increases in lipid synthesis can occur. Cell nitrogen is then apparently completely bound in essential cell constituents, and carbon subsequently fixed is converted into lipid products. The findings indicate that nitrogen limitation may be useful in increasing the food quality of batch-cultured cells, but the technique has little value for continuous culture systems per se.

There is considerable evidence that fat accumulation takes place in many algae as a response to the exhaustion of the nitrogen supply in the medium (11, 13, 15, 18). Aach analyzed samples from nitrogen-limited *Chlorella* cultures at intervals during growth and reported that the fat content of the cells rose from 22% of dry weight on the 2nd day to 70% on the 25th day (1). Collyer and Fogg (6) found that the fatty acids of *Navicula pelliculosa* increase steadily with age, this being associated with decreasing cell nitrogen content. Growth (i.e., increase in cell number) continues slowly for some time in a medium completely depleted of nitrogen, producing cells of high fat content (9). As nitrogen depletion progresses, chlorophyll and protein contents decrease and carbohydrate content increases, followed by increases in lipid content.

The unicellular algae are being considered for use in bioregenerative life support systems for space vehicles and other closed environments (12). The algae in such systems would consume carbon dioxide, produce oxygen, and serve as a potential food source for the inhabitants. Under optimal growth conditions, however, algal tissue contains an overabundance of protein and an insufficiency of usable calories for mammalian diets. It has been proposed that variations in environmental variables might be used to overcome this difficulty (2). Myers (*personal communication*) suggested that the possibility of using a controlled nitrogen deficiency to improve the food value of algae grown in continuous-culture, gas-exchange systems warrants investigation.

The study reported here was therefore performed to determine the effects of nitrogen limitation on the composition, tissue production, and oxygen yield of algae produced in continuous culture.

MATERIALS AND METHODS

The algae used in the study were *Oocystis polymorpha* Groover and Bold and *C. sorokiniana* Shihira and Krauss. The culture system was a modified annular chamber patterned after that of Myers and Clark (16) and set up for chemostatic (continuous

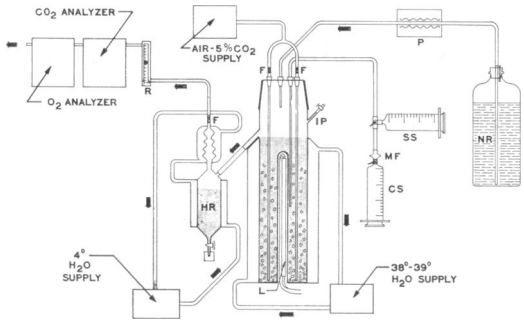

FIG. 1. *Apparatus used for the chemostatic continuous culture of Oocystis polymorpha and Chlorella sorokiniana. Abbreviations: NR, nutrient reservoir; P, peristaltic pump; SS, sample syringe; MF, membrane filter (Millipore Corp.); CS, clearing syringe, for clearing sample tube with sterile air; IP, injection port, for inoculating system through rubber tubing via hypodermic syringe; F, cotton air filter; HR, harvest reservoir; R, gas flow rotameter and regulator; L, 15-w fluorescent lamp.*

dilution-continuous overflow) operation (Fig. 1). Volume of the culture chamber was 405 ml. The chamber was illuminated externally with eight fluorescent lamps (General Electric F 42 T6 CW) and internally with one lamp (F15 T8 CW). The chamber, harvest reservoir, and all tubing were sterilized before use by autoclaving. Nutrient medium was sterilized in the nutrient reservoir by autoclaving, and the reservoir was connected into the culture system by means of "Swagelok" quick-connect fittings, the ends of which were sterilized by flaming. The chamber was gassed with air-5% CO_2, sterilized by cotton filters, at a rate of 70 ml/min. The system was inoculated by injecting, with a sterile hypodermic syringe, a dilute suspension of axenic cells through a length of sealed rubber tubing mounted on the chamber wall.

After an initial batch growth period, nutrient solution was fed into the chamber at a rate of 0.5 ml/min by means of a peristaltic pump (model 600; Harvard Apparatus Co., Dover, Mass.). The effluent cell suspension from the chamber passed by gravity flow into a chilled 1.2-liter reservoir. Effluent gas passed into the harvest reservoir, through a chilled condenser, and through a Lira 500 (Mine Safety Appliance Co., Pittsburgh, Pa.) CO_2 analyzer and a Beckman model E-2 oxygen analyzer.

Each culture was initiated with a modified Knops solution (19) containing KNO_3 as the nitrogen source at a concentration of 20 mM. After 8 to 10 days of steady-state growth, the nitrogen concentration of the input medium was reduced. Successive reductions in nitrate concentration were made at similar intervals of steady-state growth.

The cell suspension was removed from the harvest reservoir daily and stored at 0 C. Cell counts (cells/milliliter) were determined with a hemocytometer. The tissue weight per ml of suspension was determined by filtering 5 ml of suspension through a tared HA filter (Millipore Corp., Bedford, Mass., 0.45 μm), followed by drying at 70 C and weighing.

Chlorophyll content of the harvested cells was determined by methanol extraction and colorimetry (8). A 5-ml amount of culture suspension was centrifuged, and the resulting pellet was used for the assay. Per cent chlorophyll in the cell tissue was calculated by use of the dry weight determined by filtration.

To determine lipid content of the harvested cells, 40 ml of the cell suspension was centrifuged, and the supernatant fluid was discarded. The pellet was resuspended in 20 ml of chloroform-methanol (2:1), a small quantity of purified sand was added, and the suspension was sonic-treated for 3 min with a Branson Sonifier (model 8125; Heat Systems Co., Great Neck, N.Y.). The preparation was then filtered through glass-fiber filter paper. The residue was discarded and the filtrate was transferred to a centrifuge tube. Water (10 ml) was added and the mixture was centrifuged briefly. The upper (water) phase was drawn off and discarded. After an additional water washing, the chloroform-lipid layer was transferred to a tared weighing bottle, dried at 45 C, and weighed. The results were expressed as the percentage of dry weight. To reduce the effect of variations in chlorophyll, which has little food value (3), the percentage of chlorophyll was subtracted from the total percentage of chloroform-extractable material and the result was taken to be per cent lipid.

To verify the results of the total lipid assays, the fatty acid fraction of *Chlorella* tissue from the various nitrate input levels was analyzed by gas-liquid chromatography. Samples (1 g) of lyophilized tissue were extracted with chloroform-methanol (2:1) by sonic treatment. The extracts were partitioned with saturated sodium chloride solution, washed, and methylated by the boron trifluoride-methanol technique (14).

The fatty acid methyl esters were purified by column chromatography on silicic acid and analyzed with a series 5000 gas chromatograph (Barber-Colman Co., Rockford, Ill.), with a 15% diethylene glycol succinate (DEGS) column and a hydrogen flame ionization detector.

Nitrogen content of the cell tissue was determined by the micro-Kjeldahl technique. A 50-ml amount of cell suspension was centrifuged; the pellet was washed once and then transferred to a digestion flask with a minimal amount of water. The procedure thereafter followed the usual Kjeldahl technique.

Residual nitrate in the effluent medium was monitored by analysis of the culture supernatant fluid with the phenol-disulfonic acid technique (4). The effluent medium was also examined periodically by atomic absorption spectrophotometry to ascertain that reductions in influent potassium nitrate never resulted in potassium limitation.

The effluent cell suspension remaining after samples had been taken for the above analyses was centrifuged, washed, frozen, and lyophilized. The lyophilized tissue from each nitrogen level was pooled and ground to pass a 40-mesh screen with a Wiley mill. Caloric values of triplicate 1-g samples from each nitrogen

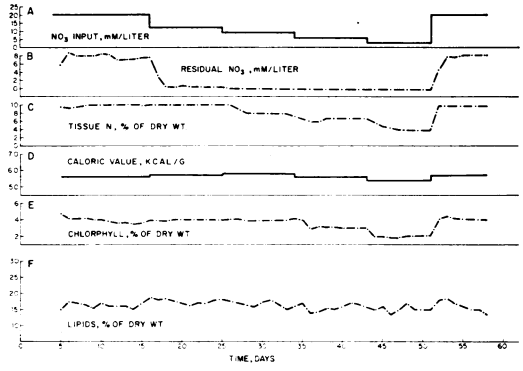

FIG. 2. *Effects of nitrogen limitation on the nitrogen content, caloric value, chlorophyll content, and lipid content (minus chlorophyll) of Oocystis polymorpha grown in continuous culture.*

level were determined by means of an oxygen bomb calorimeter (Parr Instrument Co., Moline, Ill.). Carbon, hydrogen, and nitrogen content of the lyophilized tissue was determined with a CHN analyzer (F & M model 185; Hewlett-Packard Co., Avondale, Pa.). Ash content was determined by ashing a known weight of tissue in a muffle furnace.

Carbon dioxide uptake and oxygen evolution were calculated by difference between input and output values for the culture system. Carbon dioxide values were monitored and recorded continuously, whereas oxygen readings were made twice daily.

To afford a comparison of the continuously cultured cells with cells grown in batch culture, *C. sorokiniana* was inoculated into 1-liter flasks containing 500 ml of sterile Knop's solution having a nitrate concentration of 5 mmoles/liter. The flasks were sponge-stoppered and incubated in a shaker-incubator (model R-27; New Brunswick Scientific Co., New Brunswick, N.J.). The incubator was maintained at 39 C and gassed continuously with air-5% CO_2. Flasks were removed periodically and subjected to nitrogen and fatty acid analysis by the methods given above.

RESULTS

The effects of nitrogen limitation on the growth, composition, and gas exchange of *O. polymorpha* in continuous culture are summarized in Figs. 2 and 3. Nitrate concentration of the influent medium was reduced stepwise from 20 mmoles/liter to 12, 9, 6, and 3 mmoles/liter and then was returned to 20 mmoles/liter. At concentrations of 20 and 12 mmoles/liter, nitrogen was not limiting, as evidenced by residual nitrate remaining in the supernatant fluid of the effluent cell suspension (Fig. 2B). At lesser input concentrations, however, no nitrate was recovered in the effluent medium and the nitrogen content of the cells declined from 10% to a low of 4% of the cell weight (Fig. 2C).

Chlorophyll content of the harvested tissue was constant until nitrate input had been reduced to 6 mmoles/liter (Fig. 2E). It then declined from 4 to 3% of tissue weight, and at 3 mmoles of nitrate per liter a further decline to 2% occurred. At these lower nitrate concentrations, the cells in the chamber were conspicuously chlorotic, having progressed from dark green to yellow-green in color.

No significant change in lipid content was encountered at any point in the experiment (Fig. 2F). Caloric values for the tissue ranged from 5.81 kcal/g at a nitrate input of 9 mmoles/liter to 5.37 kcal/g at a level of 3 mmoles/liter (Fig. 2D).

Oxygen evolution, carbon dioxide uptake, and tissue production all declined sharply at the lower nitrate concentrations (Fig. 3A, B, C). Cell number, as measured in both the chamber and in the harvest suspension, decreased gradually with decreases in nitrogen input.

At the 3 mmoles/liter level of nitrate input, the culture was near the "washout" point; i.e., any further reduction in the growth rate would have resulted in the loss of the culture. Similar results were obtained with continuously cultured *C. sorokiniana* (Table 1). As nitrogen input was decreased, growth rate, carbon dioxide uptake, oxygen evolution, and chlorophyll content de-

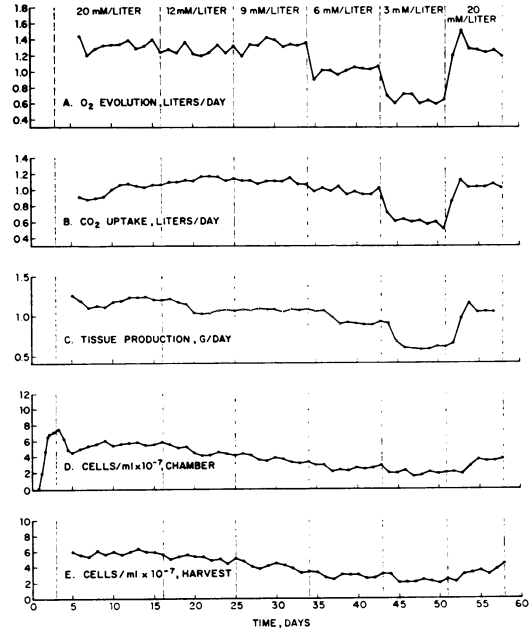

FIG. 3. *Effects of nitrogen limitation on the oxygen evolution, carbon dioxide uptake, tissue production, and cell number of continuously cultured Oocystis polymorpha.*

TABLE 1. *Effects of nitrogen limitation on the composition, yield, and gas exchange of Chlorella sorokiniana in continuous culture*

Determination	Influent nitrate concn (mmole/liter)		
	20	10	5
Residual nitrate (mmole/liter)	4.6	0	0
N (% of tissue dry wt)	10.1	7.3	5.6
Caloric value (kcal/g)	5.60	5.16	4.89
Chlorophyll (% dry wt)	5.1	2.0	1.3
Lipid (% dry wt)	16.3	15.2	14.6
O_2 evolution (liters/day)	1.93	1.57	0.77
CO_2 uptake (liter/day)	1.75	1.32	0.68
Tissue production (g/day)	1.59	1.31	0.97

TABLE 2. *Composition of total fatty acids from Chlorella tissue produced in various conditions of nitrogen supply*[a]

Determination	Influent NO_3 concn (mmole/liter)		
	20	10	5
Fatty acid (mg/g of tissue)	52.08	55.82	43.26
Fatty acid concn (mole%)			
14:0	2.1	T[b]	1.7
16:0	21.2	22.2	24.0
16:1	6.5	4.3	6.0
16:2	14.1	9.1	6.7
18:0	T	T	T
16:3	11.1	12.9	12.9
18:1	2.1	3.9	3.6
18:2	23.2	23.5	20.4
18:3	19.8	24.2	24.7

[a] Gas chromatography conditions were as follows: column 6 ft (1.8 m) by 0.25 inch (0.63 cm) outer diameter, 15% DEGS on acid-washed chromosorb W; He carrier gas flow rate 50 cc/min; column temperature 181 C; detector gases optimized for maximal response.

[b] Trace.

clined sharply, whereas slight decreases in caloric value and lipid content were evident.

The results of the fatty acid analyses (Table 2) paralleled the total lipid data. There were no significant changes in the total amounts of fatty acids present, and the relatively slight changes in the molar composition of the fatty acid fraction were such that they could be accounted for by the moderately increased light intensity caused by reduction of culture density (17). C, H, and N analyses and determination of the ash content of the lyophilized tissue permitted calculation of the tissue oxygen content by difference. Oxygen content increased with nitrogen limitation (Table 3). Calculation of the *R* values indicated that the degree of reduction of the tissue declined somewhat with *Chlorella* and was essentially unchanged for *Oocystis*, thereby confirming the results of the caloric value determinations.

In contrast to the continuous cultures, flask-cultured cells exhibited pronounced changes in the quantity and composition of the fatty acid fraction with increasing age and nitrogen limitation (Table 4). Molar composition of the fatty acid fraction was markedly different from that of the continuous culture (Table 2), even on the 2nd day after inoculation before nitrogen became limiting. This was anticipated and can be accounted for by differences in unsaturated acid levels caused by different light intensities inherent to the batch and continuous-culture systems (17). It is notable, however, that the total fatty acid concentration of tissue rose from 57.0 to a high of 137.8 mg/g of tissue, an increase of 142%. The subsequent decline in fatty acid concentration reflects the gradual deterioration of the cultures with age.

DISCUSSION

The results indicate that nitrogen limitation has little value as a technique for enhancing the food quality of algae in continuous culture. Carbon dioxide fixation and oxygen evolution are reduced too drastically for the starvation process to be a part of any gas-exchange system, and growth may even be reduced too much for the economical production of tissue in a separate "food production" module.

Previous studies on nitrogen limitation in algae have been performed with "batch" cultures. Such cultures involve the use of a determinate volume of medium, in which nutrients are not replenished. If light, carbon dioxide, and other nutrients are not limiting, the cells continue growing, although less rapidly, after the medium becomes depleted of nitrogen. At some point in time after depletion of the medium nitrogen, all of the cellular nitrogen is apparently utilized in enzymes and essential cell structures. Any carbon dioxide subsequently fixed is therefore converted into carbohydrates or fats rather than into protein. This was demonstrated in the flask cultures grown in this study (Table 4), in which lipid content reached a maximum when cell nitrogen was 3.0% of dry weight.

In the chemostatic (i.e., continuous dilution, continuous harvest) system used here, the reproductive rate of such starved cells would be less than the rate at which cells are harvested from the system, and the culture would be lost. In a

TABLE 3. *Effects of nitrogen limitation on the elemental composition of Oocystis polymorpha and Chlorella sorokiniana in continuous culture*

Determination	*Oocystis polymorpha* (NO_3 input, mmole/liter)				*Chlorella sorokiniana* (NO_3 input, mmole/liter)		
	20	12	9	6[a]	20	10	5
C	44.1[b]	46.3	46.1	45.1	47.7	43.3	41.8
H	6.6	6.8	7.3	7.0	7.0	6.8	6.8
N	10.0	8.1	6.8	4.0	10.1	7.3	5.6
Ash	6.4	5.5	4.7	4.6	5.5	7.5	7.0
O	32.9	33.3	35.1	39.3	29.7	35.1	38.8
R value	36.8[c]	36.4	38.2	35.8	48.6	36.5	34.0

[a] Tissue from nitrate at a level of 3 mmoles/liter was not sufficient to permit these analyses to be run on that treatment.

[b] Values for C, H, N, ash, and O represent per cent of dry tissue weight.

[c] $R = [(\%\ C \times 2.664) + (\%\ H \times 7.936) - 0 \times 100]/398.9$ (18).

TABLE 4. *Fatty acid concentration of nitrogen-limited, flask-cultured Chlorella*

Determination	Culture age (days)				
	2	4	6	8	10
Residual NO_3 (mmole/liter)	1.0	0.0	0.0	0.0	0.0
Tissue N (%)	7.9	3.6	3.0	3.7	4.5
Fatty acid (mg/g of tissue)	57.0	86.2	137.8	107.5	52.8
Fatty acid concn. (mole %)					
14:0	2.0	1.1	1.0	1.1	1.9
16:0	26.0	23.4	21.1	35.4	52.0
16:1	6.0	7.9	7.3	8.4	14.7
16:2	14.4	13.0	13.7	10.3	4.1
18:0	1.2	1.9	1.3	2.0	3.7
16:3	5.6	4.9	4.9	4.2	1.9
18:1	8.2	9.3	8.9	4.9	5.9
18:2	28.8	31.3	34.7	28.2	13.3
18:3	8.7	7.2	7.2	5.5	2.5

turbidostatic system (in which medium input and harvest are controlled by dilution of the culture to a constant optical density), there would be no danger of culture loss, but the reduction in productivity would be comparable. At the low rates of nitrogen input required for lipid enhancement, the turbidostat would become in essence a batch or "holding" culture.

The increases in cellular oxygen and decreases in *R* values with nitrogen limitation (Table 3) suggest a moderate increase in carbohydrate content. Although these changes do not appear to be great enough to modify the food value of the cells, comprehensive carbohydrate analyses or feeding trials would be needed to permit definite conclusions on this point. The tissue available was not sufficient to allow such tests in this study.

Alternate possibilities for lipid enhancement do exist. Although the fact that, in this study, *Oocystis* and *Chlorella* exhibited similar responses to nitrogen limitation would appear to reduce the possibility, it may be feasible to select strains or species with a greater propensity toward fat formation. Russian workers have reported some success in screening strains for increased cysteine and methionine content (10), and potassium starvation is said to increase lipid content (7). It may also be possible to grow cells to maximal density under optimal nutrient conditions, centrifuge them, and resuspend them in a nitrogen-free medium for a lipid-enhancement batch-culture period. The practicality of this technique is presently under investigation in this laboratory.

The nature of the process by which the increased lipid synthesis is induced and the drastic changes which occur in the fatty acid fraction with increasing limitation (Table 4) seem to be of considerable interest in basic studies on lipid metabolism.

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